REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-01-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
2006	Journal Article	

4. TITLE AND SUBTITLE

Functional imaging of dolphin brain metabolism and blood flow

5a. CONTRACT NUMBER

N66001-05-C-0040

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHORS

Sam Ridgway[1,2]
James Finneran, Don Carder, Mandy Keogh, William Van Bonn, and Cynthia Smith[1]
Miriam Scadeng, David Dubowitz, Robert Mattrey, and Carl Hoh[2]
Dorian Houser[3]

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

[1] SSC San Diego
Marine Mammal Program
53560 Hull Street
San Diego, CA 92152-5001

[2] UCSD
School of Medicine
9500 Gilman Drive
La Jolla, CA 92093

[3] BIOMIMETICA
7951 Shantung Drive
Santee, CA 92071

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution is unlimited. US Govt JOINT WORK.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This report documents the first use of magnetic resonance images (MRIs) of living dolphins to register functional brain scans, allowing for the exploration of potential mechanisms of unihemispheric sleep. Diazepam has been shown to induce unihemispheric slow waves (USW), therefore we used functional imaging of dolphins with and without diazepam to observe hemispheric differences in brain metabolism and blood flow. MRIs were used to register functional brain scans with single photon emission computed tomography (SPECT) and positron emission tomography (PET) in trained dolphins. Scans using SPECT revealed unihemispheric blood flow reduction following diazepam doses greater than 0.55mg kg-1 for these 180-200 kg animals. Scans using PET revealed hemispheric differences in brain glucose consumption when scans with and without diazepam were compared. The findings suggest that unihemispheric reduction in blood flow and glucose metabolism in the hemisphere showing USW are important features of unihemispheric sleep.

Functional scans may also help to elucidate the degree of hemispheric laterality of sensory and motor systems as well as in neurotransmitter or molecular mechanisms of unihemispheric sleep in delphinoid cetaceans. The findings also demonstrate the potential value of functional scans to explore other aspects of dolphin brain physiology as well as pathology.

Published in *The Journal of Experimental Biology*, Vol. 209, 2006, pp. 2902-2910.

15. SUBJECT TERMS

dolphin	diazepam	PET scan	slow wave
Tursiops	SPECT scan	brain	hemisphere autonomy
functional imaging	MRI scan	unihemispheric sleep	

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			James J. Finneran
U	U	U	UU	9	19B. TELEPHONE NUMBER *(Include area code)* (619) 767-4098

The Journal of Experimental Biology 209, 2902-2910
Published by The Company of Biologists 2006
doi:10.1242/jeb.02348

Functional imaging of dolphin brain metabolism and blood flow

Sam Ridgway[1,2,*], Dorian Houser[3], James Finneran[1], Don Carder[1], Mandy Keogh[1], William Van Bonn[1], Cynthia Smith[1], Miriam Scadeng[2], David Dubowitz[2], Robert Mattrey[2] and Carl Hoh[2]

[1]SPAWAR Systems Center San Diego, Division 235, 53560 Hull Street, San Diego, CA 92152-5001, USA, [2]School of Medicine, University of California, San Diego, CA 92093, USA and [3]BIOMIMETICA, 7951 Shantung Drive, Santee, CA 92071, USA

*Author for correspondence at address 2 (e-mail: sridgway@UCSD.edu)

Accepted 25 May 2006

Summary

This report documents the first use of magnetic resonance images (MRIs) of living dolphins to register functional brain scans, allowing for the exploration of potential mechanisms of unihemispheric sleep. Diazepam has been shown to induce unihemispheric slow waves (USW), therefore we used functional imaging of dolphins with and without diazepam to observe hemispheric differences in brain metabolism and blood flow. MRIs were used to register functional brain scans with single photon emission computed tomography (SPECT) and positron emission tomography (PET) in trained dolphins. Scans using SPECT revealed unihemispheric blood flow reduction following diazepam doses greater than 0.55 mg kg^{-1} for these 180–200 kg animals. Scans using PET revealed hemispheric differences in brain glucose consumption when scans with and without diazepam were compared. The findings suggest that unihemispheric reduction in blood flow and glucose metabolism in the hemisphere showing USW are important features of unihemispheric sleep.

Functional scans may also help to elucidate the degree of hemispheric laterality of sensory and motor systems as well as in neurotransmitter or molecular mechanisms of unihemispheric sleep in delphinoid cetaceans. The findings also demonstrate the potential value of functional scans to explore other aspects of dolphin brain physiology as well as pathology.

Key words: dolphin, *Tursiops*, functional imaging, diazepam, SPECT scan, MRI scan, PET scan, brain, unihemispheric sleep, slow wave, hemisphere autonomy.

Introduction

Dolphins and related small whales in the delphinoid cetacean family have shown slow wave sleep (SWS) electroencephalograms (EEG) in one brain hemisphere while producing waking EEG in the other (Serafetinides et al., 1970; Mukhametov et al., 1977; Mukhametov, 1984; Mukhametov, 1987; Ridgway, 2002; Lyamin et al., 2001; Lyamin et al., 2004). Left and right hemispheres alternate SWS by some unknown mechanism. Several physiological and anatomical observations suggest a degree of dolphin brain hemispheric independence. These observations include independent eye movement and closure (McCormick, 1969; Dawson et al., 1981; Lyamin et al., 2001; Lyamin et al., 2004), observations of behavior in nocturnal rest periods (Flanigan, Jr, 1974; Goley, 1999), a small corpus callosum (Tarpley and Ridgway, 1994), complete crossing of the nerves at the optic chiasm (Tarpley et al., 1994), and absence of an arterial Circle of Willis (McFarland et al., 1979). What triggers one hemisphere to go into SWS while the other hemisphere often displays an EEG indistinguishable from that of an awake animal remains to be determined.

Only once have investigators explored hemispheric physiology beyond recording EEG and other electrophysiological signs. The study of Koval'zon and Mukhametov was aimed at determining if brain temperature cycled with SWS (Koval'zon and Mukhametov, 1982). The authors studied four Black Sea bottlenose dolphins (*Tursiops truncatus*) and one harbor porpoise (*Phocoena phocoena*). Two thermisters were implanted in each animal – one in the auditory cortex of each cerebral hemisphere. During SWS, the temperature of the hemisphere displaying SWS was ~1°C lower than the opposite hemisphere, which displayed an EEG indistinguishable from the waking state. Koval'zon and Mukhametov concluded that a unihemispheric reduction of metabolic heat produced by neurons and glia accompanied the SWS (Koval'zon and Mukhametov, 1982).

Later, Mukhametov noted that the benzodiazepine tranquilizer diazepam induced 'dolphin unihemispheric SWS

in its most vivid form' (Mukhametov, 1987). Diazepam binds to $GABA_A$ receptors and a change in the sensitivity of $GABA_A$ receptors is one mechanism that might be involved in dolphin unihemispheric SWS. There is ample evidence that GABA plays a major role in sleep regulation in land mammals (Ali et al., 1999; Xi et al., 1999; Gallopin et al., 2000; Koop et al., 2004). Garey et al. (Garey et al., 1989) determined that the quantitative distribution of GABA neurons in the Black Sea porpoise (*Phocoena phocoena*) within the visual cortex is similar to that in land mammals.

It can be said that bottlenose dolphins and their close relatives in the cetacean family, Delphinidae, have large brains and have reached the zenith of cetacean brain development (Marino, 1998; Ridgway, 1999; Marino et al., 2004). Modern morphomolecular studies of fixed material have begun to reveal information relative to the neurochemistry of some regions of the dolphin brain (cf. Hof et al., 1995; Glezer et al., 1998; Manger et al., 2003; Manger et al., 2004). However, non-invasive means of investigating this large and highly organized brain in the living animal have been quite limited and there is little understanding of the neurotransmitter and neuro-modulator distribution in the dolphin brain as a whole. Prior to our recent studies (Houser et al., 2004), live cetacean scans were limited to one computed tomography CT) study of a pygmy sperm whale with a sinus abscess (Tristan et al., 2001). Houser et al. (Houser et al., 2004) expanded the use of medical imaging modalities on live cetaceans to include functional scanning (SPECT and PET) and coupled the images obtained with these scans to structural imagery obtained *via* CT. To investigate brain function in context of the finer anatomy of the brain, CT imaging of dolphin anatomy must be replaced by an imaging modality sensitive to soft tissue. MRI permits detail of soft tissues to be discerned, but the application of MRI to living cetaceans has yet to be reported.

The combination of functional imaging with soft and hard tissue structural imaging will permit *in vivo* assessments of dolphin brain functional anatomy. The information obtained from such scans will yield invaluable information on dolphin brain physiology, making possible the understanding of some of the apparently distinctive capabilities of dolphins. Such capabilities include their excellent SONAR system, the tactile sensitivity of their skin, the ability of the brain to withstand hypoxia during diving, acoustic communication, underwater vision, and how dolphins sleep at sea. Additionally, the combined imaging modalities can increase both our understanding of how various medications affect brain chemistry and our ability to employ imaging techniques in the diagnoses of illness in the dolphin brain.

Here we report results of the first functional scans of the dolphin brain registered to MR images obtained in the same animals. The functional scans, SPECT and PET, were collected with and without the administration of diazepam to induce SWS. SPECT scans were used to monitor cerebral blood flow and PET scans were used to estimate brain glucose metabolism via the uptake of a glucose analog. Differences in treatment and non-treatment scans were used to describe the physiology of unihemispheric SWS as a function of the brain's specific anatomy by co-registration to MRI scans. The results provide the first ever indication of localized and regional variations in brain metabolism and blood flow resulting from the induction of unihemispheric SWS.

Table 1. *Details of dolphin subjects*

Dolphin	Sex	Age (years)	Mass (kg)	Length (cm)	Scan type
WEN	M	21	196	252	MRI, SPECT, PET
OLY	M	21	182	239	PET
FLP	M	26	225	256	SPECT
MAY	M	30	209	260	MRI

Materials and methods

Procedures for scans

Three live, adult males (WEN, OLY and FLP) and one *post mortem*, adult male (MAY) bottlenose dolphins (*Tursiops truncatus* Montagu) were used in this study (Table 1). This study includes two MRIs (WEN and MAY), three SPECT scans (2 WEN and 1 FLP), and four PET scans (2 WEN and 2 OLY).

Prior to this study the animals were trained to slide out of the water onto a padded transport mat (Fig. 1). Functional scans (SPECT and PET) were either baseline (no diazepam prior to ligand injection) or diazepam test scan. For scans under the influence of diazepam, the animal was given 0.55–0.60 mg kg^{-1} in a fish 1 h prior to their removal from the water. Taking a lead from Mukhametov's observation that

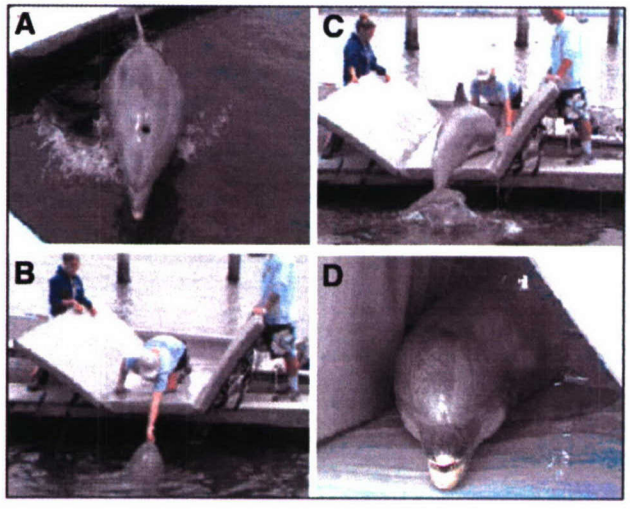

Fig. 1. A trained dolphin slides out of the water onto a padded transport mat. (A) Dolphin swims around its bay enclosure. (B) The dolphin is signaled to station in front of the trainer. (C) The dolphin slides out onto the padded transport mat. (D) The padded sides of the transport mat are brought together so that the dolphin is secure in the mat with the lateral walls up and fastened.

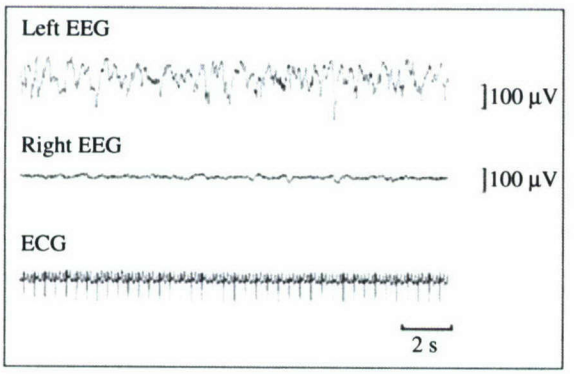

Fig. 2. Unihemispheric slow waves appearing on the left brain hemisphere EEG 1 h after a dose of 0.55 mg kg^{-1} of diazepam. ECG, electrocardiogram.

diazepam could produce unihemispheric slow waves (Mukhametov, 1987), we determined that this amount of diazepam was just over the threshold dosage for producing signs of unihemispheric EEG slow waves in our dolphins (Fig. 2). In a separate preliminary study, the dosage was as determined by EEG telemetry (D70-EEE, Transoma Medical, Aden Hills, MN, USA) from needle electrodes (25-gauge Neuroline, Ambu, Denmark) placed to the skull – two electrodes over each hemisphere while the dolphin was resting quietly, without external stimulation and with negligible movement, in our veterinary clinic (Ridgway, 2002). There was an interval of at least 2 weeks between each diazepam dose to minimize the animal's potential for developing a tolerance to diazepam.

Each scan procedure began with the dolphin voluntarily sliding onto the transport mat (Fig. 1). After a short ride to the dolphin veterinary clinic, the animal was injected with the ligand [^{99m}Tc-bicisate for SPECT, ^{18}F-2-fluoro-2-deoxyglucose (FDG) for PET] into the central circulation through the common brachiocephalic vein (Fig. 3) under ultrasound guidance. After injection of the ligand, the dolphin was kept in a darkened area and had movement or stimulation minimized during the ligand brain uptake period. The animal, with trainers and attending veterinarian, was then transported by covered truck to the nearby imaging facility where the scan was begun within 2 h after ligand injection.

Since the tables used by the various scanners were not built to take the mass of a dolphin (180–230 kg), a special table was constructed to fit over the human table and hold all of the dolphin's mass as the animal was placed in the scanner. All of the animals were trained to remain still while out of the water. An animal trainer was present during the scans, stationed just in front of the animal (Fig. 4). The animal was kept moist during the scans by sponging its skin with water. The scanners were protected from the water by placing thin plastic sheeting under the dolphin. Respiration, body temperature (*via* rectal thermometer), and electrocardiogram were monitored during the procedure. Dolphins returned to their enclosures within 4 h of being removed from the water for the procedure (Fig. 1).

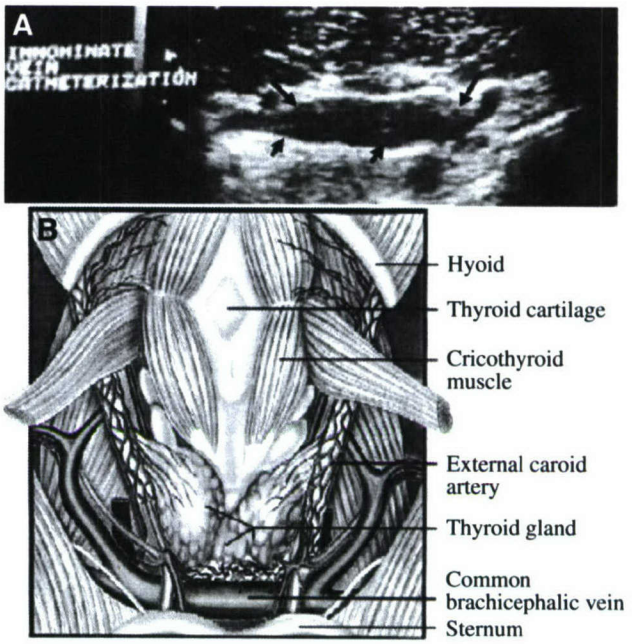

Fig. 3. (A) Ultrasound of the vetrum of the dolphin's neck showing the common brachiocephalic vein (outlined by arrows) where the ligand was injected (in the past this vein was sometimes called innominate). (B) Illustration from a dissection showing the anatomy of the area.

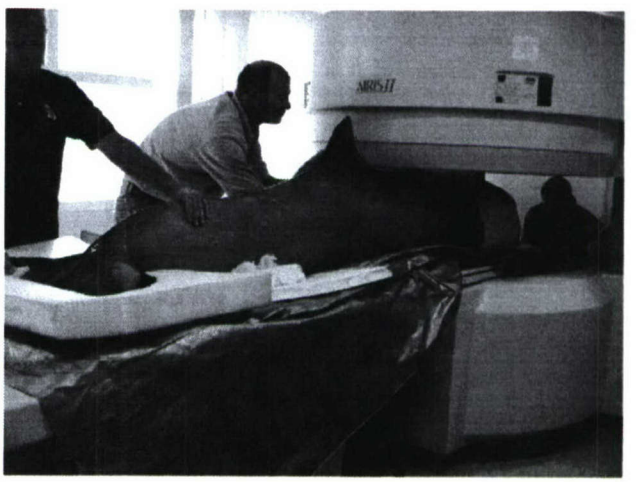

Fig. 4. MRI scan of dolphin WEN with a 0.5 T AIRIS II open scanner. The animal rests on a thin rubber pad. The forward half of the body rests on the scanner while the rear body rests on a special table constructed for dolphin scans. Attendants stabilize the animal and keep it wet while the trainer is stationed in front of the dolphin. A standard human back coil is placed around the dolphin's head immediately behind the blowhole to cover the area of the brain.

SPECT scans

Dolphins WEN and FLP were scanned using a SPECT scanner (ADAC Forte SPECT camera, Milpitas, CA, USA)

following an administration of 50 mCi (1850 MBq) of ^{99m}Tc-bicisate (Neurolite®), a radiopharmaceutical used to map blood flow and to diagnose vascular abnormalities of the brain (Itoh et al., 2001; Laliberte et al., 2004; Kusaka et al., 2005). More details on the scan procedure are published elsewhere (Houser et al., 2004). In the control (non-diazepam) scan, the dolphin was not given diazepam until 20 min after the Neurolite® injection so that the animal was not under the influence of the diazepam during the radiopharmaceutical uptake period. In the test scan, the diazepam was given 1 h before the injection of Neurolite® so that the animal would be under the influence of diazepam while the Neurolite® was being taken up by the brain. Blood analysis showed significant levels of circulating diazepam 1 h after oral administration (data not shown).

PET scans

The same non-diazepam/diazepam protocol was followed when dolphins WEN and OLY were administered 20 mCi (740 MBq) of ^{18}F-2-fluoro-2-deoxyglucose (FDG). FDG is an analog of glucose and is often used in PET imaging to estimate glucose uptake by the brain. FDG was given ~2 h prior to each of four scans (one with and one without prior diazepam each for WEN and OLY) to map relative metabolic activity within the brain. As in the SPECT procedure, the animal was kept in a quiet, darkened room for 20 min after injection of the ligand. The dolphin was then transported, as outlined above, to the facility where the PET scans were conducted. Images were acquired on a Seimens HR+ PET scanner (Knoxville, TN, USA) with the dolphin on the same specially engineered table as used in the SPECT scan. A 5-min transmission scan was first acquired for attenuation correction. The emission scan consisted of eight frames of 4 min acquisitions to allow for repetition in case of any subject movement. This resulted in a total scan time of approximately 37 min. The scan images were converted from the ECAT7.2 format to DICOM 3.0 for further processing (see also Houser et al., 2004).

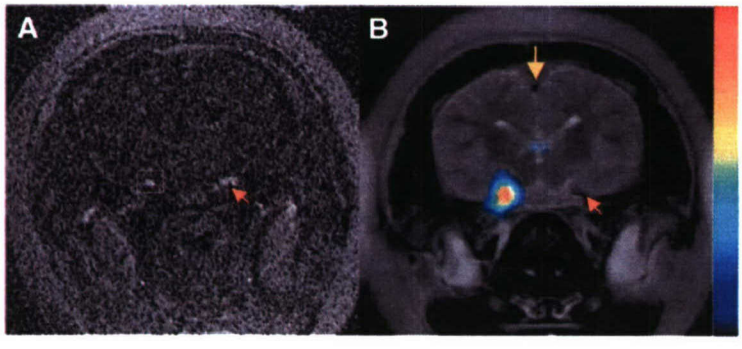

Fig. 5. (A) Time-of-flight magnetic resonance image (MRI) from dolphin WEN demonstrating anterior blood flow through arteries of the brain outlined by the box. (B) Fused MRI and SPECT images from co-registered scans made in the same dolphin. The colored region corresponds to a reduction in blood flow; the color bar indicates the relative degree of blood flow reduction with red indicating maximum reductions in blood flow. The yellow arrow indicates the central venous sinus. The red arrows indicate the homolog cerebral artery on the right side of the brain that does not show blood flow reduction. Registration, image analysis and fusion were performed with ANALYZE.

MRI scan

The first MRI scan ever done on a live dolphin was accomplished with the dolphin WEN (Figs 4, 5). The dolphin had been exposed to the recorded sounds of the MRI scanner over 10 training periods during the month before the actual scan. The dolphin received oral diazepam (0.55 mg kg^{-1} body mass) 2 h before the scan. MRI data were collected on a Hitachi Airis II, 0.5 Tesla (T) scanner. A T2 weighted pulse sequence was used to acquire image data in the axial plane. Data were acquired with a slice thickness of 8 mm, a slice interval of 9 mm, and FOV of 280. The repetition rate (TR) was set to 5700 ms, echo time (TE) set to 125 ms, and flip angle set to 90°. A total of 20 slices were acquired with a scan time of approximately 3.5 min. These scan slices were then used for registration of the SPECT and PET scans.

When a dolphin (MAY), not associated with this project, died of natural causes, the animal was perfused immediately after death with 4% paraformaledhyde in buffered ringer's solution. After fixing *in situ*, this brain was removed from the skull and scanned on a 3 T scanner for finer anatomical detail. Based on cranial volume measurements, the brain of MAY was of similar size to both WEN and OLY. We were not able to MRI scan subject OLY and thus registered some of the OLY scans to sections of this well-fixed *post mortem* brain. Some scans obtained from WEN were also registered to the MAY scans to show more anatomical details than were available in the 0.5 T scans of WEN.

Image analysis

The Subtraction Ictal SPECT co-registered to MRI algorithm, or SISCOM, was used to analyze variations in ^{99m}Tc-bicisate distribution and FDG uptake as a function of diazepam induced unihemispheric sleep. The SISCOM procedure capitalizes on seizure-related transient increases in regional blood flow to isolate the anatomy of the brain involved in the seizure. The algorithm is amenable to other methods of assessing variation in brain function using similar isotopic methods. In this study, SISCOM was employed to isolate focal regions of the brain that demonstrated reduced blood flow or reduced metabolism following induction of unihemispheric sleep.

Data acquired from all of the imaging modalities were processed using Analyze 5.0/6.0, created by the Biomedical Imaging Resource of the Mayo Clinic (Robb, 1999). All data were converted to AVW format (native Analyze format) and volumes made cubic (equivalent voxel dimensions) through the use of linear interpolation. Test data were co-registered to the control data from the same respective scan type and animal using the normalized mutual information (NMI) voxel

matching algorithm. The control volume and transformed test volume were then segmented for creation of binary masks. Using the 'Morphology' module of Analyze, thresholds were applied to the volumes so that isotope activity within the brain was isolated from surrounding tissues. The volumes were then segmented and exported as a binary volume. Holes within the binary volumes were filled utilizing a 2D processing algorithm applied in the transverse, coronal and sagittal planes, and then once again in the transverse plane. The resultant control and treatment binary volumes were then multiplied together to form a binary mask common to the two volumes.

Binary masks common to the SPECT volume were multiplied by the control and co-registered test volumes, respectively, to generate masked control and co-registered treatment volumes. The information in these volumes corresponded only to combined estimates of voxels within the brain. The mean value of all non-zero voxels was determined for the masked control and masked co-registered treatment volume and mean values were subsequently used to normalize the respective volumes to a normalized mean of 100. The normalized co-registered treatment volume was then subtracted from the normalized control volume, resulting in a mean voxel value near zero, and the standard deviation of voxel values within the subtraction volume was calculated.

Voxels corresponding to the brain were segmented from the MRI volume and the volume passed through an inhomogeneity filter. The control volume was then co-registered to the segmented MRI volume utilizing the 'Surface Matching' algorithm within Analyze. The registration was fine-tuned through manual controls and the resultant transform matrix was applied to the control volume, co-registered treatment volume, and subtraction volume. Once co-registered to the MRI volume, each of the SPECT and PET volumes were color-mapped to an 8-bit color scale and fused to the MRI to permit the overall pattern of blood flow or metabolic activity to be observed, as well as activity of focal regions within the brain to be isolated.

Local reductions in blood flow following diazepam treatment were visualized by fusing to the MRI only those voxels within the subtraction SPECT volume with values more than two standard deviations below the mean value of the subtraction volume. Similarly, local reductions in metabolism were visualized by fusing to the MRI only those voxels within the subtraction PET volume with values more than two standard deviations below the mean value of the subtraction volume. For both the PET and SPECT scans, values more than two standard deviations below the mean corresponded to a greater than 95% reduction in isotope distribution and activity, relative to the control. Thus, the anatomy to which these voxels are mapped correspond to regions of reduced blood flow (SPECT) and regions of reduced glucose uptake (PET).

Results

Functional SPECT scans

Processing of SPECT images revealed an area of reduced blood flow around a major artery in the left hemisphere (Fig. 5). The light area at the center of the square on the left shows the left middle spinal meningeal artery, a major supply to the left brain (Fig. 5A). On Fig. 5B are overlaid regions of decreased blood flow from two previous SPECT scans imaged with 50 mCi (1850 MBq) of technetium (Tc-99m) biscisate (Neurolite®). One SPECT scan was under the influence of 0.55 mg kg^{-1} of diazepam while the other was not. The colored areas show regions of a least two standard deviations of reduction in blood flow.

Functional PET scans

Pet images from dolphin WEN are shown in Figs 6 and 7. Four sample frames, left and right sagittal, coronal and axial from Dolphin WEN without diazepam treatment are shown in Fig. 6. In Fig. 7 are different coronal, axial and sagittal sections showing the reduction in glucose consumption in the diazepam scan in specific areas. In these scan comparisons from Dolphin WEN, areas of metabolic reduction were most pronounced in the right hemisphere and especially in the right posterior cortex (Fig. 7N,O), right insular cortex (Fig. 7B,M), cerebellum (Fig. 7B,C,G,N,O), and notably in the right locus coeruleus (Fig. 7F). However, some areas of marked metabolic reduction appeared in the left cortex, especially in frontal areas (Fig. 7A,E).

Fig. 8 shows raw scan sections from Dolphin OLY as seen with the program PET VIEWER® (©Tim Van den Wyngaert). Four scan sections in the left column (Fig. 8A–D) were taken

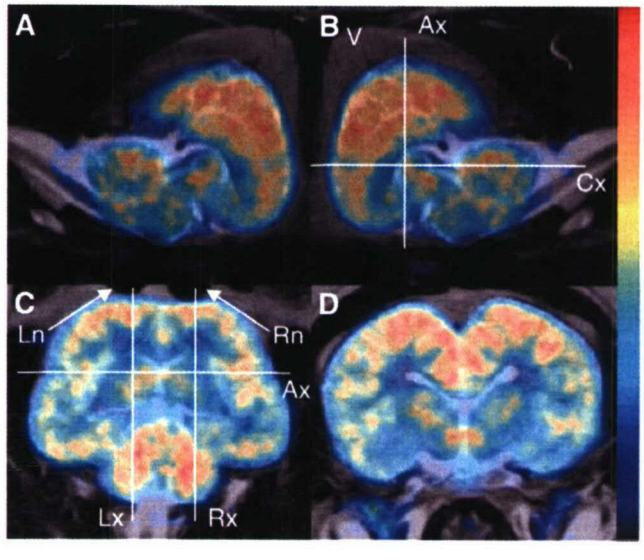

Fig. 6. Four planes from a control FDG PET scan of dolphin WEN registered to a 0.5 T scan of the same animal. No diazepam was given before the ligand injection. (A) left sagittal section (B) right sagittal section showing the vertex of the skull (V) and the planes of the axial section (Ax) and coronal section (Cx). (C) The coronal section showing the left nasal cavity (Ln), the right nasal cavity (Rn), and the planes of the axial section (Ax), left sagittal section (Lx), and the right sagittal section (Rx). (D) Axial section. The color bar indicates relative degree of glucose metabolism with red indicating maximum.

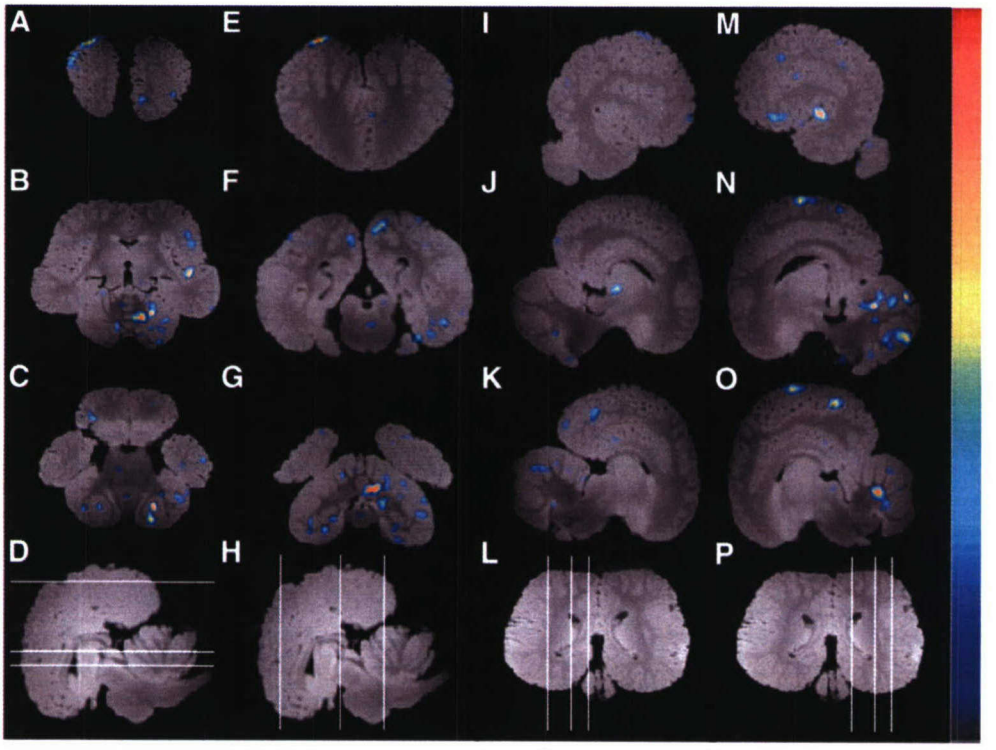

Fig. 7. A subtraction of a diazepam scan from a non-diazepam scan of dolphin WEN registered to twelve 3.0 T MRI sections of dolphin MAY. From left to right: coronal (A–C), axial (E–G), left sagittal (I–K) and right sagittal (M–O). Lines on scan sections (D,H,L,P) at the bottom of each column indicate the plane of sections from the three scans in the same column above. The color indicates the relative degree of metabolic reduction in the diazepam scan with red indicating maximum reductions in glucose consumption. In this series there is an overall reduction in metabolism in the right brain hemisphere; however, there are some areas of lower metabolism in the left hemisphere, especially in frontal areas.

without prior diazepam (control scan) while the four scan sections in the center column (Fig. 8E–H) were taken with the dolphin under the influence of 0.60 mg kg^{-1} of diazepam. Sections from the diazepam scan (center column) show greater asymmetry than sections from the control scan (left column). Some selected sections (Fig. 8J–L) registered to the 3 T MRIs of Dolphin MAY and sliced on the oblique (I) to show the hippocampus (section K), reveal metabolic reduction (compared to control scan) on the left side. There is discernable metabolic reduction in the left hippocampus.

Discussion

Since this was the first MRI study of a living dolphin, we were concerned about the animal's potential magnetic sensitivity (Bauer et al., 1985). The animal remained quite still while in the magnet and showed no apparent response during the scan. Examination of the scans revealed no indication of magnetite; however, since granules of magnetite are usually no more that 50 μm in diameter the grains, if present, could have been too small to see with our MRI system.

This investigation of functional imaging focused not only on developing methodology for live dolphin imaging but also on diazepam, known to enhance sleep in humans and laboratory animals (Sierra et al., 1997; Echizenya et al., 2003; Koop et al., 2004). Diazepam also produces unihemispheric sleep in dolphins (Mukhametov, 1984; Mukhametov, 1987). Our observations of unihemispheric SWS after diazepam dosages of 0.55 or 0.60 mg kg^{-1} body mass is supportive of the previous findings. In the present study, diazepam caused a reduction in blood flow to one brain hemisphere as demonstrated by SPECT imaging (Fig. 5).

The locus coeruleus (LC) is a key structure modulating sleep and wakefulness in humans and laboratory animals (Nitz and Siegel, 1997). Immunohistochemistry has been employed to characterize the dolphin LC (Manger et al., 2003). There are no specific specializations in the dolphin LC that set it apart from the structure of other mammals as might have been expected in a mammal with a large brain and the ability to sleep unihemispherically. In terrestrial mammals studied, the firing rate of LC neurons slows during SWS (Nitz and Siegel, 1997; Manger et al., 2003). It is particularly noteworthy that there was a significant reduction in metabolism of the right LC areas in our study as shown in Fig. 7F. Our findings lend support to the suggestion (Manger et al., 2003) that dolphin LC neurons must fire at a constant rate, slowing in only one side of the brain during SWS, to maintain muscle tone for swimming and thermoregulating in cold water.

While it is known that diazepam may cause hypothermia in laboratory mammals (Dowden et al., 1999), hypothermia as measured by rectal temperature was not observed in this study. However, it is possible that regional temperature reductions could be present. For example one brain hemisphere could be slightly cooler and the other slightly warmer. Dolphins have numerous retia mirabila that are known to function as counter-current heat-exchangers to retain metabolic heat within certain regions of the body (Rommel et al., 1993; Heyning and Mead, 1997). The blood supply to the brain comes through a vast retial network in the dorsum of the thorax not through the internal carotids (McFarland et al., 1979).

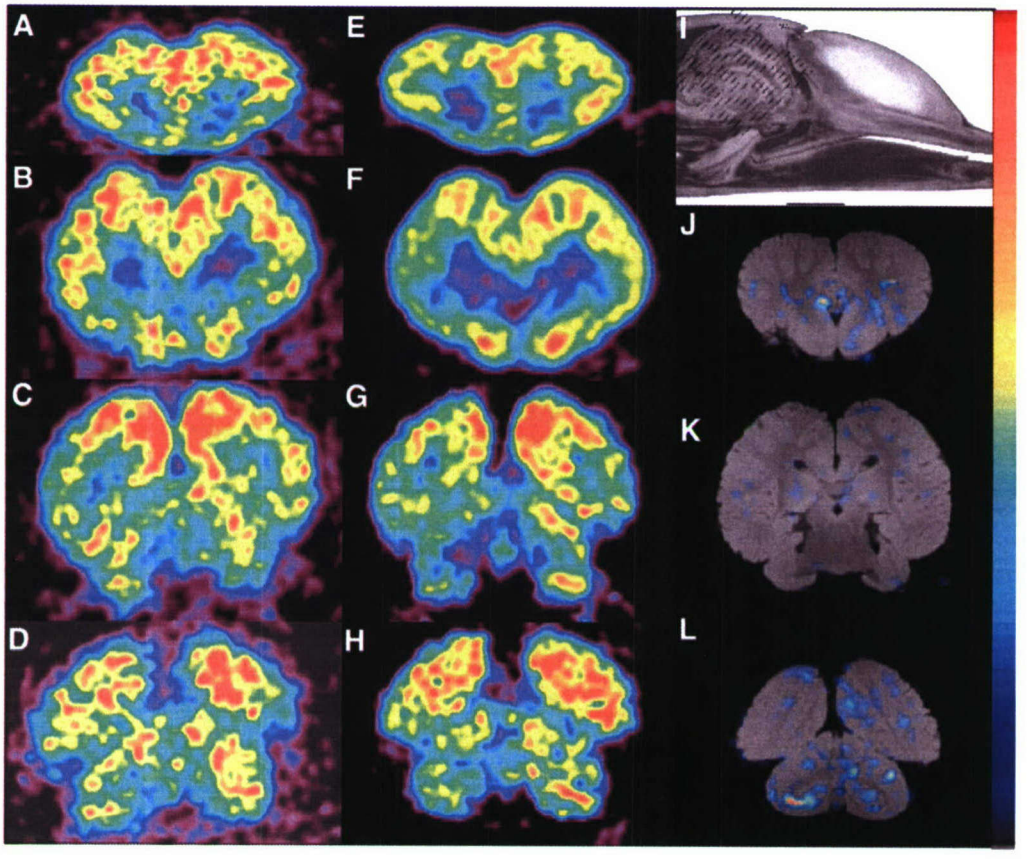

Fig. 8. Comparison of four sections each of two different scans of dolphin OLY. The left column (A–D) shows a scan without diazepam while the center column shows sections from a scan with diazepam (E–H). Overall, metabolism is lower in the left hemisphere. The color bar indicates the relative degree of glucose metabolism in sections A–H with red indicating maximum. The right hand column shows oblique axial scans (as indicated in the upper right, section I) of dolphin MAY's MRI, to which have been registered the difference volumes between the two scans. In sections J–L, the colored regions correspond to a reduction in metabolism in the diazepam scan; the color indicates the relative degree of metabolic reduction with red indicating maximum reductions in glucose consumption.

Our studies suggest that cerebral blood flow reduction may be a controlling factor in the temperature reduction observed by Koval'zon and Mukhametov during unihemispheric slow wave sleep (Koval'zon and Mukhametov, 1982). In mammals, brain temperature may be influenced by three factors: (1) the temperature of blood flowing to the brain, (2) the rate of cerebral blood flow, and (3) the metabolic heat production of neurons and glia. Reduced cerebral blood flow and therefore reduced glucose supply likely will affect regional brain temperature and metabolic heat production. Furthermore, these factors may impact $GABA_A$ receptor sensitivity to diazepam (Patel et al., 2005; Garey et al., 1989) such that a reciprocal effect between the hemispheres could be created so that the active or 'non-sleeping' hemisphere would have a raised threshold for sleep.

The development of the capability to functionally scan dolphins and the finding of unihemispheric diazepam effects has suggested a hypothesis of hemispheric defense. That is, the dolphin brain hemispheres cycle between two brain states that we will call 'State 0' and 'State 1.' In 'State 0' that brain hemisphere may be awake and fully alert or it may sleep. The opposite hemisphere in 'State 1' is usually awake and is defended against sleep by physiological mechanisms as yet not completely understood.

The ability to have EEG slow waves in one brain hemisphere (Mukhametov et al., 1977; Mukhametov, 1984; Mukhametov, 1987; Ridgway, 2002) while maintaining an ability to swim and a degree of vigilance (Lilly, 1964) may not be the only advantage of the unihemispheric physiology observed in the dolphin brain. Deep and prolonged diving is important to the foraging success of most dolphin populations (Evans, 1971; Ponganis et al., 2003). The dolphin's large and active brain, especially the huge and elaborate neocortex, is a considerable metabolic expense (Robin, 1973; Hockett, 1978; McFarland et al., 1979). Alveolar gas tensions after long dives by dolphins was suggested to indicate that the dolphin brain might be capable of short periods of anaerobic metabolism (Ridgway et al., 1969), a capability lacking, or much reduced, in adult land mammals that have been studied (anaerobic brain metabolism has been demonstrated in seals in the later stages of a maximal

dive) (Kerem et al., 1971; Simon et al., 1974). For the dolphin, brain oxygen consumption could also be reduced by unihemispheric vasoconstriction, reduced blood flow and glucose consumption, as observed with our SPECT and PET scans. The ability to partially 'shut down' or at least reduce oxygen and glucose consumption in a major portion of the brain might be an advantage to a dolphin making repetitive, prolonged feeding dives.

This study has shown that dolphins can be trained to participate in non-invasive scans that can be useful in understanding their brain blood flow, metabolism and many other aspects of their specialized physiology and anatomy. Functional scans may help to elucidate the degree of laterality of sensory and motor systems. Scans may reveal neurotransmitter or molecular mechanisms of physiology that cannot be explored in any other way. The techniques developed here can also be useful in detecting pathology and in the clinical care of these interesting and valuable animals.

All experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committees of the Navy Marine Mammal Program, Space and Naval Warfare Systems Center, San Diego, CA, USA and the School of Medicine, University of California, San Diego. We thank J. Corbeil and the staff of Molecular Imaging Incorporated and the Department of Nuclear Medicine at the University of California, San Diego Medical Center for their assistance in performing scans. We also thank the Biomedical Imaging Resource of the Mayo Clinic, Rochester for their assistance with ANALYZE. Drs Eric Jensen and Chris Dold, Joel Baumbaugh (Health Physics) and the animal care and training staff of the Space and Naval Warfare Systems Center (SSC San Diego), were critical for their training, transport and care of the dolphin subjects. We especially thank Mark Todd and Tricia Kamolnick for their technical expertise in training and their continuous efforts with the dolphin subjects. We appreciate the encouragement of Drs John Carney, Lisa Eley, Amy Kruse and Brett Giroir. We also thank two anonymous reviewers who made very helpful suggestions. This project supported by the Defense Advanced Research Projects Agency (DARPA) under SSC San Diego Contract N66001-05-C-0040.

References

Ali, M., Jha, S. K., Kaur, S. and Mallick, B. N. (1999). Role of GABA-A receptor in the preoptic area in the regulation of sleep-wakefulness and rapid eye movement sleep. *Neurosci. Res.* **33**, 245-250.

Bauer, G. B., Fuller, M., Perry, A., Dunn, R. and Zoeger, J. (1985). Magnetoreception and biomineralization of magnetite in cetaceans. In *Magnetite Biomineralization and Magnetoreception in Organisms* (ed. J. L. Kirschvink, D. S. Jones and B. J. MacFadden), pp. 489-507. New York: Plenum Press.

Dawson, W. W., Carder, D. A., Ridgway, S. H. and Schmeisser, E. T. (1981). Synchrony of dolphin eye movements and their power density spectra. *Comp. Biochem. Physiol.* **68A**, 443-449.

Dowden, J., Reid, C., Dooley, P. and Corbett, D. (1999). Diazepam-induced neuroprotection: dissociating the effects of hypothermia following global ischemia. *Brain Res.* **22**, 1-6.

Echizenya, M., Mishima, K., Satoh, K., Kusanagi, H., Sekine, A., Ohkubo, T., Shimizu, T. and Hishikawa, Y. (2003). Heat loss, sleepiness, and impaired performance after diazepam administration in humans. *Neuropsychopharmacology* **28**, 1198-1206.

Evans, W. B. (1971). Orientation behavior of delphinids: radio telemetric studies. *Ann. NY Acad. Sci.* **188**, 142-160.

Flanigan, W. F., Jr (1974). Nocturnal behavior of captive small cetaceans 1, the Bottlenosed Porpoise *Tursiops truncatus*. *Sleep Res.* **3**, 84.

Gallopin, T., Fort, P., Eggermann, E., Cauli, B., Luppi, P. H., Rossier, J., Audinat, E., Muhlethaler, M. and Serafin, M. (2000). Identification of sleep-promoting neurons in vitro. *Nature* **404**, 992-995.

Garey, L. J., Takacs, J., Revishchin, A. V. and Hamori, J. (1989). Quantitative distribution of GABA-immunoreactive neurons in cetacean visual cortex is similar to that of land mammals. *Brain Res.* **485**, 278-284.

Glezer, I. I., Hof, P. R. and Morgane, P. J. (1998). Comparative analysis of calcium-binding protein-immunoreactive neuronal populations in the auditory and visual systems of the bottlenose dolphin (*Tursiops truncatus*) and the macaque monkey (*Macaca fascicularis*). *J. Chem. Neuroanat.* **15**, 203-237.

Goley, P. D. (1999). Behavioral aspects of sleep in Pacific white-sided dolphins (*Lagenorhynchus obliquidens*, Gill 1865). *Mar. Mamm. Sci.* **15**, 1054-1064.

Heyning, J. and Mead, J. G. (1997). Thermoregulation in the mouths of feeding Gray Whales. *Science* **278**, 1138-1139.

Hockett, C. F. (1978). In search of Jove's bow. *Am. Speech* **53**, 243-313.

Hof, P. R., Glezer, I. I., Revishchin, A. V., Bouras, C., Charnay, Y. and Morgane, P. J. (1995). Distribution of dopaminergic fibers and neurons in visual and auditory cortices of the harbor porpoise and pilot whale. *Brain Res. Bull.* **36**, 275-284.

Houser, D. S., Finneran, J., Carder, D., Van Bonn, W., Smith, C., Hoh, C., Mattrey, R. and Ridgway, S. (2004). Structural and functional imaging of bottlenose dolphin (*Tursiops truncatus*) cranial anatomy. *J. Exp. Biol.* **207**, 3657-3665.

Itoh, K., Korogi, Y., Tomiguchi, S., Takahashi, M., Okajima, T. and Sato, H. (2001). Cerebellar blood flow in methylmercury poisoning (Minamata disease). *Neuroradiology* **43**, 279-284.

Kerem, D., Elsner, R. and Wright, J. (1971). Anaerobic metabolism in the brain of the harbor seal during the late stages of a maximum dive. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 384.

Koop, C., Rudolph, M., Low, K. and Tobler, I. (2004). Modulation of rhythmic brain activity by diazepam: $GABA_A$ receptor subtype and state specificity. *Proc. Natl. Acad. Sci. USA* **101**, 3674-3679.

Koval'zon, B. M. and Mukhametov, L. M. (1982). Temperature fluctuations of the dolphin brain corresponding to unihemisphere slow-wave sleep. *Zh. Evol. Biokhim. Fiziol.* **18**, 307-309.

Kusaka, T., Ijichi, S., Yamamoto, Y. and Nishiyama, Y. (2005). Changes in cerebral glucose metabolism in newborn infants with cerebral infarction. *Pediatr. Neurol.* **32**, 46-49.

Laliberte, J. F., Meunier, J., Mignotte, M. and Soucy, J. P. (2004). Detection of diffuse abnormal perfusion in SPECT using a normal brain atlas. *Neuroimage* **23**, 561-568.

Lilly, J. C. (1964). Animals in aquatic environments: adaptation of mammals to the ocean. In *Handbook of Physiology – Environment* (ed. D. B. Dill, E. F. Adolph and G. C. Wilber), pp. 741-747. New York: John Wiley and Sons.

Lyamin, O. I., Mukhametov, L. M., Siegel, J. M., Nazarenko, E. M., Polyakova, I. G. and Shpak, O. V. (2001). Correlation between 'unihemispheric' slow wave sleep and the state of eyes in a beluga whale. *Sleep* **24**, A40-A41.

Lyamin, O. I., Mukhametov, L. M. and Siegel, J. M. (2004). Relationship between sleep and eye state in Cetaceans and Pinnipeds. *Arch. Ital. Biol.* **142**, 557-568.

Manger, P. R., Ridgway, S. H. and Siegel, J. M. (2003). The locus coeruleus complex of the bottlenose dolphin (*Tursiops truncatus*) as revealed by tyrosine hydroxylase immunohistochemistry. *J. Sleep Res.* **12**, 149-155.

Manger, P. R., Fuxe, K., Ridgway, S. H. and Siegel, J. M. (2004). The distribution of morphological characteristics of catecholaminergic cells in the diencephalon and midbrain of the bottlenose dolphin (*Tursiops truncatus*). *Brain Behav. Evol.* **64**, 42-60.

Marino, L. (1998). A comparison of encephalization between odontocete cetaceans and anthropoid primates. *Brain Behav. Evol.* **51**, 230-238.

Marino, L., McShea, D. W. and Uhen, M. D. (2004). Origin and evolution of large brains in toothed whales. *Anat. Rec. A Discov. Mol. Cell Evol. Biol.* **281**, 1247-1255.

McCormick, J. G. (1969). Relationship of sleep, respiration, and anesthesia in the porpoise: a preliminary report. *Proc. Natl. Acad. Sci. USA* **62**, 697-703.

McFarland, W. L., Jacobs, M. S. and Morgane, P. J. (1979). Blood supply to the brain of the dolphin, *Tursiops truncatus*, with comparative observations on special aspects of the cerebrovascular supply of other vertebrates. *Neurosci. Biobehav. Rev.* Suppl. **1**, 93.

Mukhametov, L. M. (1984). Sleep in marine mammals. *Exp. Brain Res.* Suppl. **8**, 227-238.

Mukhametov, L. M. (1987). Unihemispheric slow-wave sleep in the Amazonian dolphin, *Inia geoffrensis*. *Neurosci. Lett.* **79**, 128-132.

Mukhametov, L. M., Supin, A. Y. and Polyakova, I. G. (1977). Interhemispheric asymmetry of the electroencephalographic sleep patterns in dolphins. *Brain Res.* **134**, 581-584.

Nitz, D. and Siegel, J. M. (1997). GABA release in the locus coeruleus as a function of sleep/wake state. *Neuroscience* **78**, 795-801.

Patel, A. B., de Graaf, R. A., Mason, G. F., Rothman, D. L., Shulman, R. G. and Behar, K. L. (2005). The contribution of GABA to glutamate/glutamine cycling and energy metabolism in the rat cortex *in vivo*. *Proc. Natl. Acad. Sci. USA* **102**, 5588-5593.

Ponganis, P. J., Kooyman, G. L. and Ridgway, S. H. (2003). Comparative diving physiology. In *Bennett and Elliott's Physiology and Medicine of Diving* (ed. A. O. Brubakk and T. S. Neuman), pp. 211-226. London: Harcourt.

Ridgway, S. H. (1999). The cetacean central nervous system. In *Encyclopedia of Neuroscience*. 2nd edn (ed. G. Adelman and B. Smith), pp. 352-357. New York: Springer-Verlag.

Ridgway, S. H. (2002). Asymmetry and symmetry in brain waves from dolphin left and right hemispheres: some observations after anesthesia, during quiescent hanging behavior, and during visual obstruction. *Brain Behav. Evol.* **60**, 265-274.

Ridgway, S. H., Scronce, B. L. and Kanwisher, J. (1969). Respiration and deep diving in the bottlenose porpoise. *Science* **166**, 1651-1654.

Robb, R. A. (1999). *Biomedical Imaging, Visualization and Analysis*. New York: John Wiley and Sons.

Robin, E. (1973). The evolutionary advantages of being stupid. *Perspect. Biol. Med.* **16**, 369-379.

Rommel, S. A., Pabst, D. A. and McLellan, W. A. (1993). Functional morphology of the vascular plexuses associated with the cetacean uterus. *Anat. Rec.* **237**, 538-546.

Serafetinides, E. A., Shurley, J. T. and Brooks, R. E. (1970). Electroencephalogram of the pilot whale, *Globicephala scammoni*, in wakefulness and sleep: lateralization aspects. *Int. J. Psychobiol.* **2**, 129-133.

Sierra, J. C., Luna-Villegas, G., Buela-Casal, G. and Fernandez-Guardiola, A. (1997). The assessment of residual effects of a single dose of diazepam on visually-defined EEG patterns. *J. Psychopharmacol.* **11**, 367-372.

Simon, L. M., Robin, E. D., Elsner, R., Van Kessel, A. and Theodore, J. (1974). A biochemical basis for differences in maximal diving time in aquatic mammals. *Comp. Biochem. Physiol.* **47B**, 209-215.

Tarpley, R. J. and Ridgway, S. H. (1994). Corpus callosum size in delphinid cetaceans. *Brain Behav. Evol.* **44**, 156-165.

Tarpley, R. J., Gelderd, J. B., Bauserman, S. and Ridgway, S. H. (1994). Dolphin peripheral visual pathway in chronic unilateral ocular atrophy: complete decussation apparent. *J. Morphol.* **222**, 91-102.

Tristan, T., Pelton, P. and Ewing, R. (2001). Computerized tomography of a sinus abscess in a pygmy sperm whale (*Kogia breviceps*). *IAAAM Proc.* **32**, 43-44.

Xi, M. C., Morales, F. R. and Chase, M. H. (1999). Evidence that wakefulness and REM sleep are controlled by a GABAergic pontine mechanism. *J. Neurophysiol.* **82**, 2015-2019.